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Effects of Hyperoxia and Caffeine on the Expression of Fragile Site at Xq27.3

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To enhance the cytogenetic expression of the fragile X chromosome, we studied the effects of hyperoxia and caffeine on the induction of fragile Xq27.3. A lymphoblastoid cell line (GM 06912) derived from a fragile X male proband was cultured in RPMI 1640 containing 16% dialyzed fetal calf serum. The cells were synchronously subjected to one of 3 different atmospheric oxygen tensions (21%, 21.3 kPa, normoxic; 40%, 40.5 kPa, hyperoxic; or 60%, 60.8 kPa, hyperoxic) during the last 24 hours of the 72 hour culture, immediately after the addition of 2′-deoxy-5-fluorouridine (FUdR) at 25 ng/ml. To study the enhancing effect of caffeine, with or without hyperoxia, a second set of cultures was additionally subjected to caffeine (2.5 mM) during the last 6 hours of the culture.

When the fragility of hyperoxic cells (38.1 kPa dissolved oxygen) was compared to that of normoxic control cells (13.3 kPa dissolved oxygen), the difference was significant (*P* < 0.05). These data suggest that there is a mean increase in the fragile Xq27.3 expressivity as the dissolved oxygen tension increases. Additionally, we observed that caffeine, with or without hyperoxia, significantly (*P* < 0.05) suppressed the expression of the fragile X site in this lymphoblastoid cell line. © 1996 Wiley-Liss, Inc.*

KEY WORDS: fragile Xq27.3, hyperoxia, caffeine, lymphoblastoid cell line

INTRODUCTION

The diagnosis of fragile X syndrome (Martin-Bell syndrome) is based on the association of a mental retardation syndrome with the expression of chromosomal fragility at Xq27.3. There are several factors which influence the cytogenetic expression of the fragile X site. Although genetic factors control the fragile X expression, other identifiable nongenetic factors are known to be involved [Hecht et al., 1986; Fisch et al., 1991; Verkerk et al., 1991]. Those which exert considerable control over the frequency of fragile X expression are generally associated with cytogenetic culture procedures, such as cell density [Krawczun et al., 1986], the number of cells analyzed [Fisch et al., 1991], tissue culture medium [Sutherland, 1979], and levels of folic acid and thymidylate synthase activity [Glover et al., 1986; Sutherland et al., 1985]. Enhanced expression can be induced by inhibitors of folic acid such as methotrexate and by inhibitors of thymidylate synthetase, such as 2′-deoxy-5-fluorouridine (FUdR), both of which prevent the formation of deoxythymidine monophosphate. These facts suggest that reducing the amount of thymidine available for incorporation into DNA is a crucial factor for expression of the fragile site [Glover et al., 1986; Sutherland et al., 1985]. Caffeine, which has a synergistic effect with a variety of mutagens/carcinogens, was also found to enhance the expression of fragile X when used in conjunction with FUdR [Yunis and Soreng, 1984]. However, much of the early work using caffeine was questionable because the existence of the common fragile site at Xq27.2 (FRAXD) was confused with the fragile Xq27.3 [FRAXA; Sutherland and Baker, 1990; Rao et al., 1988; Yunis et al., 1987; Ledbetter et al., 1986a,b; Glover et al., 1986; Abruzzo et al., 1986; Yunis and Soreng, 1984].

Increased site-specific Chinese hamster chromosomal fragility was reported in Chinese hamster ovary cells adapted to hyperoxic culture conditions [Gille et al., 1989]. This finding prompted our laboratory to investigate the effect of hyperoxic culture conditions on

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the expression of fragility at human Xq27.3, under the influence of FUdR or FUdR plus caffeine.

MATERIALS AND METHODS

A lymphoblastoid cell line (GM 06912) derived from a male fragile X proband with an established percentage (17%) of fragile X expressivity was obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). The lymphoblasts were cultured for 3 days at 37°C in RPMI 1640 (HEPES buffered to pH 7.4) with 16% dialyzed fetal calf serum (both obtained from Gibco/BRL, Grand Island, NY) as described by Jenkins et al. [1990].

Flasks (4 for each oxygen tension) were each seeded with 2×10^6 cells and cultured at 37°C in 5% CO_2 for 48 hours. The cultures were then treated with FUdR (25 ng/ml) and the pH was adjusted to 7.4. The culture flasks, with loosened caps, were then transferred to controlled atmosphere culture (CAC) chambers (Bellco Biotechnology, Vineland, NJ). Four flasks were placed in each CAC chamber, which was flushed with the appropriate gas mixture [Joenje and Oostra, 1983, 1986]. The gas mixtures used were 21% O_2 (normoxic/ambient, test control), 40% O_2 (hyperoxic), and 60% O_2 (hyperoxic). All gas mixtures contained 5% CO_2 with balance nitrogen (Roberts Oxygen, Rockville, MD). See Table I for final dissolved oxygen tensions. The final dissolved oxygen tension was reached within 90 minutes of exposure [unpublished data].

After incubating for an additional 24 hours in the designated oxygen environment, the flasks were removed from each CAC chamber and the caps were immediately tightened to prevent changes in dissolved oxygen tension. The pH, pCO_2, and pO_2 of each flask were determined without delay. To do so, a sample of the culture medium from the bottom of the flask was removed using a long needle attached to an insulin syringe. The sample was immediately injected into a Corning 170 Blood Gas Analyzer (Corning Medical, Medfield, MA). Shortly after the dissolved gas and pH analyses, the cultures were simultaneously subjected to standard cytogenetic harvesting procedure.

In those cultures treated with FUdR plus caffeine, the caffeine (final concentration of 2.5 mM) was added 18 hours after the addition of FUdR [Yunis and Soreng, 1984]. To accomplish this, the CAC chamber was opened, 0.25 ml 0.1 M caffeine in deionized injection-grade water was added to the appropriate flasks, and the caps were left loose as before [Budavari, 1988]. The CAC chamber was resealed and flushed with the designated gas mixture. No addition was made to control flasks which contained FUdR only. Incubation was continued at 37°C for the remaining 6 hours. Analyses were accomplished in the same manner as above.

The harvested cells from all the flasks at each oxygen tension were pooled in each experiment. Chromosome spreads were made and coded to enable observers to read the spreads in a blinded manner. The slides were aged at 65°C for 3 days and the chromosome spreads were GTG banded through standard cytogenetic procedure. More than 100 cells were examined from each pool. The experiments were repeated 3 times. Thus, for each oxygen tension, a total of more than 300 chromosome spreads were analyzed (more than 100 cells per experiment for each oxygen tension). In the first set of experiments, the recorded status of the Xq27.3 site was confirmed by a second observer. The percentage of the fragile X expression was calculated for each oxygen tension. The data were analyzed using an independent t test [Hoel, 1976]. The results were considered significantly different if $P < 0.05$.

RESULTS

The postculture media pH for each culture condition is given in Table I. The pH of the preculture media was 7.4. As expected, the pH decreased with cell proliferation. There were no significant differences in the final pH due to oxygen tension. However, there were statistically significant differences ($P < 0.05$) observed in the final pH when caffeine-containing cultures were compared to those without caffeine at the same oxygen tension. It should be noted that this difference in pH did not exceed the 0.15 pH unit. Thus the pHs of caffeine-containing cultures were consistently lower than the pHs of the cultures which did not contain caffeine.

When cells were exposed to FUdR only, those cells cultured in 38.1 kPa dissolved oxygen showed a significant ($P < 0.05$) increase in fragile X expression when compared to cells exposed to 13.3 kPa oxygen (32.4% ± 7.3 SD vs. 17.1% ± 0.5 SD fragility as shown in Table II).

In contrast, when cells were exposed to FUdR plus caffeine, only cells exposed to 24.9 kPa dissolved oxygen demonstrated significantly more fragile X expression ($P < 0.05$) than did those cells treated with 13.3 kPa dissolved oxygen (12.7% ± 1.2 SD vs. 8.7% ± 1.7 SD fragility). The difference between fragility expressed by cells exposed to 38.1 or 13.3 kPa dissolved oxygen (13.3% ± 2.5 SD vs. 8.7% ± 1.7 SD fragility) was not significant ($P = 0.06$).

Cells exposed to caffeine had significantly ($P < 0.05$) less expression of fragility than did those cells which

TABLE I. Final Dissolved Oxygen Tension and pH of Culture Media After Incubation at Different Atmospheric Oxygen Tensions†

Atmospheric O_2 tension (%)	Atmospheric O_2 tension (kPa)	Dissolved O_2 tension (kPa)	pH (SD)	pH (SD) w/caffeine#
21 (room air)	21.3	13.3	7.00 (0.19)	6.85 (0.08)*
40	40.5	24.9	6.98 (0.16)	6.87 (0.08)*
60	60.8	38.1	6.97 (0.16)	6.87 (0.08)*

† Oxygen tensions are given in kilopascals (kPa).
* Significant difference ($P < 0.05$) when compared to corresponding culture without caffeine.

TABLE II. Mean Percentage of Fragile X Expression ± SD for Three Oxygen Exposure Groups (n = 3)

		% of cells expressing fragile-X	
Target O_2 tension (kPa)	Actual O_2 tension (kPa)	FUdR only mean (SD)	FUdR + caffeine mean (SD)#
21.3	13.3	17.1 (0.5)	8.7 (1.7)*
40.5	24.9	23.9 (5.5)	12.7 (1.2)*, **
60.8	38.1	32.4 (7.3)***	13.3 (2.5)*

* Significant difference ($P < 0.05$) when compared to cultures at corresponding oxygen tensions with no caffeine added.
** Significant difference ($P < 0.05$) when compared to the FUdR plus caffeine control.
*** Significant difference ($P < 0.05$) when compared to the control cultures in FUdR only.

were not exposed to caffeine. This was true at all 3 oxygen tensions tested (Table II).

DISCUSSION

Our data indicate that when the dissolved oxygen concentration was high (38.1 kPa), fragile X expressivity was significantly increased as compared to expressivity after exposure to 13.3 kPa dissolved oxygen (Table II). Although the increase in fragile X expressivity was not significant in cells exposed to 24.9 kPa dissolved oxygen, the data suggest that hyperoxia (38.1 kPa dissolved oxygen) during the last cell cycle enhances the expressivity of this rare, folate-sensitive fragile site at Xq27.3. Hyperoxia is known to induce the generation of reactive oxygen intermediates such as superoxide anions, hydroxyl radicals, singlet oxygen, and hydrogen peroxide [Jamieson et al., 1986]. These intermediates modify DNA, RNA, proteins, and membranes [Loeb and Cheng, 1990], leading to cell cycle delay and a spectrum of spontaneous chromosomal aberrations [Joenje and Oostra, 1983].

More than 20 products of oxygen radical-mediated damage of DNA bases have so far been identified [Poltev et al., 1993]. Therefore, various mechanisms may be considered to explain the observed increase in the fragile X expressivity under the hyperoxic conditions. The following mechanisms are among the more plausible:

1. Hyperoxia may enhance FUdR's disturbance of nucleotide precursor pools in an additive manner, and thus more effectively elicit the fragile site. FUdR competitively inhibits thymidylate synthetase and thymidine production, while oxygen acts as a substrate for ribonucleotide reductase, preventing the reduction of cytidine diphosphate to deoxycytidine diphosphate. This latter reaction is also the mechanism through which excess thymidine elicits fragile site expression [Taylor and Hagerman, 1983; Anderson, 1985; Sutherland et al., 1985; Sutherland and Baker, 1986; Rao et al., 1988; Gille et al., 1989].

2. Singlet oxygen, a major oxidative species, can specifically convert deoxyguanines contained in the CGG repeats into 8-oxyguanines [Piette, 1991]. Since 8-oxyguanines are known to cause mispair formation, ambiguous coding, cell cycle delay, single-strand breaks, and sister chromatid exchanges in human lymphocytes [Piette, 1991; Devasagayam et al., 1991; Cheng et al., 1992; Khan et al., 1992; Lutgerink et al., 1993; Poltev et al., 1993], it is also reasonable to attribute the observed fragile X enhancement to 8-oxyguanines.

We selected 38.1 kPa dissolved oxygen as the maximum oxygen tension in this study due to the projected decrease in the mitotic index at higher oxygen tensions [Gille et al., 1989; Joenje and Oostra, 1983]. We did not observe any increase in chromosomal aberrations other than the increase in the fragile X induction at the 2 higher oxygen tensions tested [Joenje and Oostra, 1983, 1986]. Sporadic, single, or multiple chromosomal breaks as well as marked reductions in mitotic index were observed only in the caffeine-treated cultures, and more so in those cultures also exposed to either 24.9 or 38.1 kPa oxygen. This is expected as caffeine has also been reported to enhance chromosomal breakage at common fragile sites [Yunis et al., 1987].

When caffeine was used in the presence of FUdR, we observed a significant reduction ($P < 0.05$) in the expression of fragility at Xq27.3 in the normoxic as well as the 2 hyperoxic cultures (Table II) as compared to cultures without caffeine. This observation may suggest that caffeine suppresses the expression of the fragile site at Xq27.3 even under hyperoxia. The observation of an overall reduced expression of fragility at Xq27.3 in our caffeine-treated studies, irrespective of the dissolved oxygen tension, seems contrary to some of the early reports since much of the early work using caffeine is questionable because the existence of the common fragile site at Xq27.2 (FRAXD) was confused with the fragile X (FRAXA) and this has been documented by Sutherland and Baker [1990]. Furthermore, they demonstrated that a reduction of approximately 0.5 pH unit in lymphocyte culture medium decreased the frequency of expression of the fragile X site from 20% to 10% [Sutherland, 1979]. However, we observed a decrease in the frequency of expression of the fragile X site equal to or greater than this when the pH reduction was only 0.15 pH unit or less, indicating that the decrease in pH alone may not be fully responsible for the observed decrease in fragility in our caffeine-treated cultures (Tables I, II). The suppressive effect of caffeine may also be through the poor mitotic yield observed in our caffeine-treated cultures and/or altered cell cycling due to poor G2 repair synthesis and condensation delay [Webb, 1992; Kihlman, 1977.

When cultures were exposed to FUdR plus caffeine, those cultured at ambient oxygen tensions (13.3 kPa dissolved oxygen) demonstrated significantly less ($P < 0.05$) fragile X expression than those cultured at 24.9 kPa dissolved oxygen (Table II). This indicates that, in spite of the caffeine's suppressive action, the fragile X site is more expressive under hyperoxia, although not maximally expressive. However, the suppressive effects of caffeine and the enhanced fragile X expression under increased (24.9 kPa and 38.1 kPa) oxygen conditions seem to somehow be interactive at those levels of hyperoxia.

The present study, although limited to a single lymphoblastoid cell line, clearly underlines the need for more extensive study of the fragile X site under hyperoxia, involving multiple cell lines with full as well as premutation. Although the augmented cytogenetic expression of the fragile X site, under an appropriate hyperoxic condition, may enhance the efficacy of the current cytogenetic diagnosis of the affected male and female individuals, molecular methods will remain the method of choice in evaluating families to identify and establish risks for unaffected carrier female and male individuals. Although molecular studies can distinguish the 3 distinct repeat sites in the region [Xq28(FRAXE), Xq28(FRAXF), and Xq27.3(FRAXA); Parrish et al., 1994], it is also important to cytogenetically show the presence of the folate-sensitive (FRAXA) fragile site and to investigate the usefulness of the augmented cytogenetic expression under hyperoxia in detecting the fragile X site in the premutation stage. This work also needs to be extended to peripheral blood lymphocyte analysis in fragile X patients and their families. Such studies may contribute to our understanding of the relationship between fragile site molecular sequences and the expression of a structural lesion on a chromosome. Moreover, the clarification of the basis for the enhanced cytogenetic expression of the fragile X site under hyperoxic conditions might also lead to a better understanding of the molecular mechanisms involved in the expression of these unique dynamic mutations.

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